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Brief report

Gene expression profiling of dedifferentiated human articular chondrocytes in monolayer culture

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SUMMARY

Objective: When primary chondrocytes are cultured in monolayer, they undergo dedifferentiation during which they lose their phenotype and their capacity to form cartilage. Dedifferentiation is an obstacle for cell therapy for cartilage degeneration. In this study, we aimed to systemically evaluate the changes in gene expression during dedifferentiation of human articular chondrocytes to identify underlying mechanisms.
Methods: RNA was isolated from monolayer-cultured primary human articular chondrocytes at serial passages. Gene expression was analyzed by microarray. Based on the microarray analysis, relevant genes and pathways were identified. Their functions in chondrocyte dedifferentiation were further investigated.
Results: In vitro expanded human chondrocytes showed progressive changes in gene expression. Strikingly, an overall decrease in total gene expression was detected, which was both gradual and cumulative. DNA methylation was in part responsible for the expression downregulation of a number of genes. Genes involved in many pathways such as the extracellular-signal-regulated kinase (ERK) and Bone morphogenetic protein (BMP) pathways exhibited significant changes in expression. Inhibition of ERK pathway did not show dramatic effects in counteracting dedifferentiation process. BMP-2 was able to decelerate the dedifferentiation and reinforce the maintenance of chondrocyte phenotype in monolayer culture.
Conclusion: Our study not only improves our knowledge of the intricate signaling network regulating maintenance of chondrocyte phenotype, but also contributes to improved chondrocyte expansion and chondrogenic performance for cell therapy.

Introduction

Articular chondrocytes maintain the articular cartilage homeostasis by synthesis of extracellular matrix (ECM) molecules including type II collagen (COL2) and proteoglycans[1]. The phenotype of differentiated chondrocytes is unstable and rapidly lost with exposure to inflammatory factors, as well as during monolayer culture[2,3]. This process is designated dedifferentiation and is characterized by the loss of ECM molecules such as COL2[4]. Autologous chondrocyte implantation (ACI) is the golden therapy for patients with focal lesions of articular cartilage[5,6]. ACI consists of removal of a small piece of intact articular cartilage, isolation and expansion of chondrocytes to obtain sufficient number of cells for implantation. Dedifferentiation of chondrocytes during *in vitro* expansion is a major obstacle in ACI. The exact mechanism of the dedifferentiation remains elusive. This study aims to provide systemic analysis of mechanisms of human articular chondrocyte dedifferentiation.

Methods, results and discussion

Human articular chondrocytes were isolated from knee joints of patients undergoing total knee replacement surgery for end stage osteoarthritis (OA) ($n = 4$, mean age ± standard deviation (SD): 62.8 ± 7.6) using 1 mg/ml collagenase II (Sigma–Aldrich) prepared in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) at 37°C overnight under agitation, and were seeded at a density of 25,000 cells/cm^2 in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen). Chondrocytes were cultured in monolayer up to passage 8 and assessed at passage 0, 2, 4, 6 and 8. Chondrocytes showed typical phenotypic changes during monolayer culture. Cells lost their round shape and became flattened (Fig. S1A). To test if dedifferentiated chondrocytes

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maintained their ability to produce cartilage-specific ECM, passage 2 and 8 chondrocytes were cultured in pellets (200,000 cells per well were seeded in a round-bottom 96-wells plate and centrifuged for 3 min at 2000 rpm to form high-density micromass cell pellets) in chondrogenic differentiation media (DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, 0.2 mM ascorbic acid 2-phosphate, 0.4 mM proline, 10 ng/ml transforming growth factor (TGF)-β1) for 3 weeks and then subjected to histological analysis. Cell pellets were fixed with 10% formalin for 15 min and embedded in paraffin using routine. Sections of 5 μm were cut and stained for sulfated glycosaminoglycans (GAGs) with alcian blue combined with counterstaining of nuclear fast red to observe nuclei. Passage 2 cells were still able to produce a cartilage-specific ECM while passage 8 cells had lost their ability to regain chondrocyte characteristics (Fig. S1B). These data suggest that after expansion for eight passages human chondrocytes have lost their capacity to produce cartilaginous matrixes at least *in vitro*.

For a comprehensive understanding of chondrocyte dedifferentiation, we analyzed global gene expression changes during monolayer culture. RNA samples from passage 0, 2, 4, 6, and 8 human articular chondrocytes of four donors were isolated using Absolutely RNA Miniprep Kit (Agilent Technologies) and subjected to microarray analysis. The Ambion Illumina total prep 96 kit was used to generate biotinylated cRNA. 750 ng of the cRNA of each sample was hybridized onto Illumina HumanHT-12 v3 Expression BeadChips. BeadChips were scanned using the Illumina iScan array scanner. Gene expression profiling was performed using Illumina's Genomestudio v. 2010.1 with default settings advised by Illumina. The raw fluorescence intensity values were normalized applying quantile normalization. Differential gene expression was analyzed using GeneSpring, version 11.5.1 (Agilent Technologies). Genes with at least a two-fold difference and significantly differentially expressed according to a one-way analysis of variance with a Benjamini–Hochberg false discovery rate correction and TukeyHSD post-hoc test, using a cut-off rate of $P = 0.05$, were selected. Gene expression levels were compared to that of passage 0 (P0). The raw and normalized data are deposited in the Gene Expression Omnibus database (GSE42235). Table S1 shows all genes with at least a two-fold change in expression at P8 compared to P0. 137 genes showed over two-fold significant change at passage 2 [Fig. 1(A)]. Over time, an increasing number of genes showed significant differences. 93 genes showed consistent changes through all passages compared to P0 [Fig. 1(A)]. Interestingly, the 10 most changed genes were almost all down-regulated during all passages as compared to P0 (Table S2). In agreement with this, the average of fold changes in gene expression levels of all significantly changed genes at each passage decreased from P2 to P8 [Fig. 1(B)]. When gene expression levels of one passage were compared to those of the previous passage (e.g., P0–P2, P2–P4, P4–P6, P6–P8), the average rate of decrease in expression level of the subsequent passage appeared constant [Fig. 1(C)], indicating that the dedifferentiation process is both gradual and cumulative.

Since more genes showed downregulation of expression during chondrocyte dedifferentiation and DNA methylation is an epigenetic mechanism for gene expression silencing implicated in chondrogenesis and cartilage degeneration[7,8], we explored the role of gene expression silencing by DNA methylation. P2 and P8 chondrocytes of one of these donors were transiently treated with the chemical analog of cytidine, 5-AzaC, inhibiting DNA methylation. RNA samples were isolated and cDNA was synthesized from total RNA with the iScript cDNA synthesis kit (Bio-Rad). quantitative polymerase chain reaction (qPCR) was performed with the MyiQ real-time PCR detection system (Bio-Rad) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as internal control. *SOX9* and *BMP2* expression was increased by DNA methylation inhibition, though *COL2A1* was not affected [Fig. 1(D)]. *MMP1*, *MMP3* and *MMP13* also showed increased expression in response to 5-AzaC treatment [Fig. 1(D)]. This suggests that, at least in this donor, expression of a set of genes is cumulatively repressed by DNA methylation or other epigenetic mechanisms contributing to gradual loss of the differentiated phenotype.

Ingenuity pathway analysis (IPA) software (Ingenuity Systems) was used to identify biological processes and pathways associated

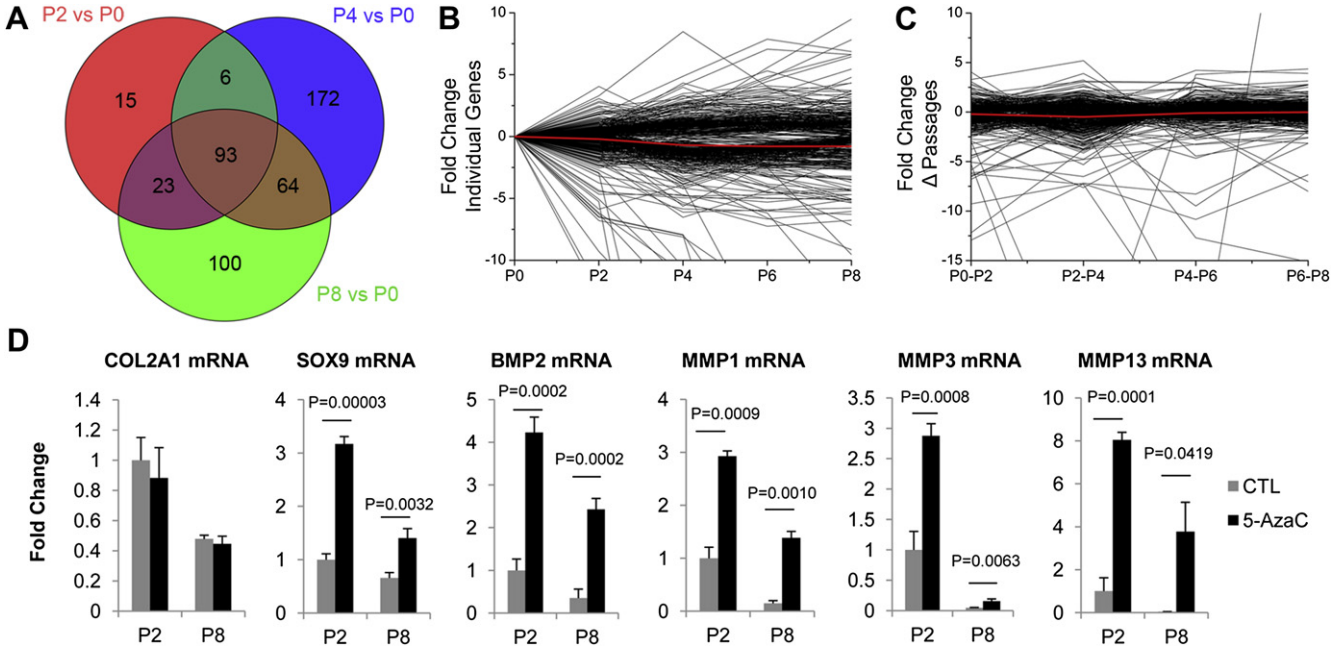

Fig. 1. Whole genome gene expression analysis of dedifferentiating chondrocytes. (A) Venn diagram depicting the number of significantly changed genes with ≥2.0 mean fold change at P2, P4 and P8 compared to P0 respectively of four donors. (B) Fold changes of all individual genes showing significant changes at each passage of four donors. Expression levels of each passage were compared to P0. The mean fold change is indicated by red line. (C) Fold changes of each gene between adjacent passages (P0–P2, P2–P4, P4–P6, P6–P8). Average of differences between passages is indicated by red line. (D) Involvement of DNA methylation in downregulation of gene expression. P2 or P8 chondrocytes were treated with 2 μM 5-AzaC for 48 h mRNA levels were measured by qPCR. Data were expressed as mean with SD and analyzed by *t*-test. *n* = triplicate cultures of one donor.

with the modulated gene expression. Significantly modulated genes were involved in multiple biological processes (Fig. S2). Notably, cellular growth and proliferation, cell death and cell cycle were significantly changed, most obvious in P4 and P6. This is consistent with a decrease in proliferation in monolayer culture[9]. Other pathways directly related to cartilage biology, such as skeletal and muscular system development and function and connective tissue development also changed. The cell morphology pathway also showed a significant change, which is in agreement with the phenotypic changes observed (Fig. S1A). Genes that exhibited significant changes in expression and are associated with these biological processes are shown in Table S3.

To validate the microarray data, expression of a number of significantly changed genes was measured by qPCR for the same RNA samples as used in microarray. All qPCR results and microarray data showed strong correlation, indicating valid and successful microarray analysis (Fig. S3). Gene expression of ECM molecules *COL2A1* and *COL9A1* decreased while versican expression increased, characterizing the chondrocyte dedifferentiation process. The chondrogenic transcription factor *SOX9* exhibited a steep reduction in expression from P0 to P2. SOX9 is the key transcription activator driving *COL2A1* expression[10] and overexpression of SOX9 enhances the ability of chondrocytes to maintain their differentiated phenotype during expansion[11]. Loss of these important cartilaginous markers may eventually lead to the loss of ability of chondrocytes to produce cartilage-specific ECM and form stable cartilage *in vivo*. Other transcription factors, receptors and extracellular ligands including bone morphogenetic protein (BMP), fibroblast growth factor (FGF), insulin-like growth factor 1 and Notch changed significantly, suggesting that chondrocyte dedifferentiation may be controlled by a complex network of pathways. The ability of chondrocytes to produce ECM is inversely correlated with the culture period. While P2 chondrocytes can still be induced to produce substantial amounts of ECM, the ability of P8 chondrocytes to produce ECM is severely compromised. This is confirmed by our observation that expression of *COL2A1* and *COL9A1* decreases in time. Other markers such as *BMP2* and *FGFR3*, which predict the capacity of expanded human chondrocytes to form stable cartilage *in vivo*[12], showed continuous decrease during dedifferentiation. Matrix degrading enzymes *MMP1*, *MMP3* and *MMP13* were down-regulated.

Network analysis based on gene expression changes between P8 and P0 chondrocytes revealed the involvement of multiple signaling pathways (Fig. S4). Key signaling mediators such as Akt, extracellular-signal-regulated kinase 1/2 (ERK1/2), PI3K, p38 MAK and nuclear factor-κB formed the cores of these networks. These signaling pathways are at the crossroads of many genes shown to be gradually but significantly altered during prolonged culturing. These mediators function as upstream or downstream effectors of these changed genes. We examined whether ERK plays a determining role in dedifferentiation. Western blot was performed to examine the

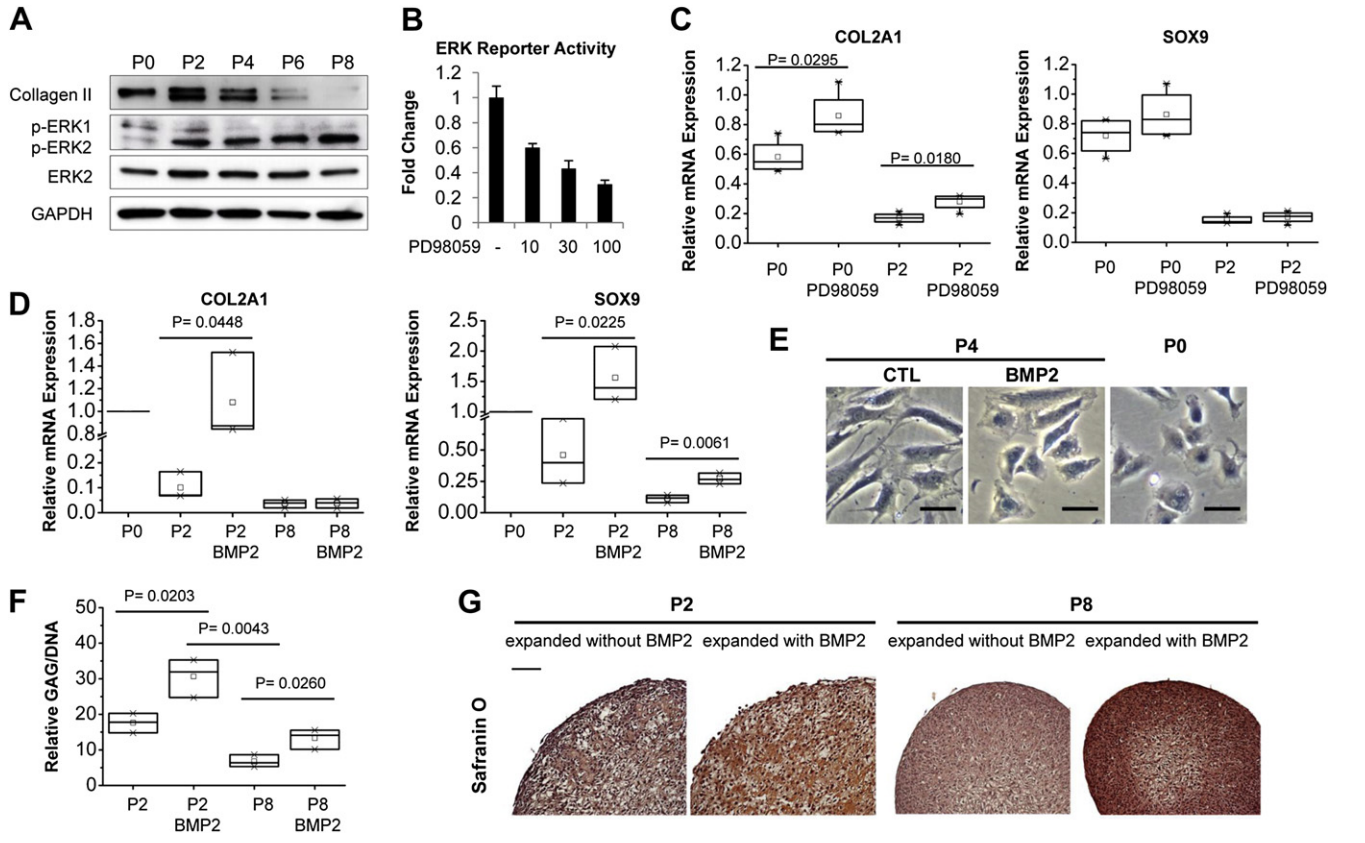

Fig. 2. Involvement of ERK and BMP-2 pathways in human chondrocyte dedifferentiation. (A) Protein levels of collagen II, phospho(p)-ERK, total ERK, and GAPDH were detected by western blot. Blots from one representative donor out of three donors are shown. (B) ERK reporter activity was measured after treatment with different concentrations of PD98059 for 24 h as indicated (in μM) in chondrocytes lentivirally transduced with luciferase promoter reporter construct. Data were expressed as mean with SD. n = triplicate cultures of one donor. (C) Human chondrocytes were cultured with or without 10 μM PD98059 from P0 until P2. mRNA expression was measured by qPCR. Data were expressed as mean with 95% confidence intervals and analyzed by t-test. $n = 3$ donors. (D) Human chondrocytes were cultured with or without 100 ng/ml BMP-2 from P0 until P8. mRNA expression levels were measured by qPCR. Data were expressed as mean with 95% confidence intervals and analyzed by t-test. $n = 3$ donors. (E) Light microscopic images of chondrocytes cultured with or without BMP-2 were taken at P4. Scale bar = 5 μm. Images from one representative donor out of three donors are shown. (F&G) P2 and P8 chondrocytes expanded with or without 100 ng/ml BMP-2 were re-differentiated in pellet culture in chondrogenic differentiation medium. (E) GAG production of chondrocyte pellets from chondrocytes expanded with or without BMP-2 was measured and normalized for DNA content. Data were expressed as mean with 95% confidence intervals and analyzed by t-test. $n = 3$ donors. (F) Histological staining of GAG/sulphated GAG in pellet sections was performed. Scale bar = 100 μm. Images from one representative donor out of three donors are shown.

chondrocyte marker COL2 and ERK protein expression. COL2 protein showed decreased expression with increasing passaging, in agreement with decreased COL2A1 mRNA expression [Fig. 2(A)]. Remarkably, in culture chondrocytes from P2 onwards started to express a smaller isoform of COL2A1 which might be a transcription variant or cleaved protein which decreased over time [Fig. 2(A)]. There was an increase in phospho-ERK2 and a decrease in phospho-ERK1, with ERK2 as the dominant form of ERK [Fig. 2(A)]. Total ERK2 did not show a substantial change during dedifferentiation. To test the function of increased ERK activity during dedifferentiation, we used the MEK1 inhibitor PD98059 which showed nearly 50% inhibition of ERK activity at 10 μM [Fig. 2(B)]. We cultured human chondrocytes from P0 to P2 in the presence of 10 μM PD98059. *COL2A1* expression was slightly but significantly increased at both P0 and P2 [Fig. 2(C)], which is consistent with previous findings[2,13]. However, the key chondrogenic transcription factor SOX9, important for the maintenance of the chondrocyte phenotype[11], was not affected by ERK inhibition [Fig. 2(C)]. Gain of function analysis using a constitutive active MEK1 has shown a role for ERK in regulating chondrocyte proliferation and negatively regulating chondrocyte hypertrophy in mice models[14]. This indicates a similar role for MEK–ERK in the regulation of chondrocyte proliferation and inhibition of the differentiation capacity of chondrocytes in prolonged monolayer culture. The weak effect of ERK inhibition suggests that it may not be the main driving factor of chondrocyte dedifferentiation. We therefore did not explore this further.

BMP-2 has been shown to induce the expression of chondrocyte-specific genes, such as *COL2A1* and *SOX9*[15]. Our microarray and qPCR data showed that *BMP2* expression decreased during dedifferentiation, implying that it may be involved in loss of chondrogenic markers and the chondrocyte phenotype. Direct stimulation of P0 human chondrocytes with BMP-2 increased the expression of *COL2A1* and *SOX9*, both of which showed decreased expression during dedifferentiation (Fig. S5A). When 100 ng/ml BMP-2 was added during extensive expansion until P8, downregulation of these genes was counteracted at P4 and decelerated at P8, except for *COL2A1* [Fig. 2(D)]. In presence of BMP-2 a reduction in cell proliferation at P2 vs control was seen, but no significant difference in proliferation rate between control and BMP-2-expanded chondrocytes at P8 was demonstrated (Fig. S5B). In addition, BMP-2 was able to reduce phenotypic changes of the chondrocytes during dedifferentiation [Fig. 2(E)]. To test whether addition of BMP-2 during culture expansion enhances the capacity of expanded chondrocytes (P2 and P8) to produce cartilage matrix, chondrocytes cultured with or without BMP-2 in monolayer were re-differentiated in pellet cultures in chondrogenic differentiation media (containing no BMP-2). GAG production was significantly higher in chondrocyte pellets formed by cells expanded in the presence of BMP-2 at both P2 and P8 [Fig. 2(F)]. Moreover, histological staining of chondrocyte pellet sections revealed that BMP-2 stimulated the production of cartilage-specific ECM [Fig. 2(G)]. Taken together, these data suggest that loss of BMP-2 expression may be an important trigger of chondrocyte dedifferentiation. Moreover, it suggests that BMP-2 signaling seems to be upstream of many other events in the dedifferentiation, especially at early stage of dedifferentiation.

Perturbation of the described signaling pathways during extended culturing provides opportunities for prevention of chondrocyte dedifferentiation and preservation of the chondrocyte phenotype, greatly improving the outcome of ACI.

Author contributions

B. Ma, J. C. H. Leijten, J. N. Post and M. Karperien were involved in conception and design. B. Ma, L. Wu and M. Kip were involved in acquisition of data. B. Ma, J. C. H. Leijten, C. A. van Blitterswijk, J. N. Post and M. Kaperien were involved in analysis and interpretation of data. B. Ma, J. C. H. Leijten, J. N. Post and M. Karperien were involved in drafting and critical revision of the article.

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Conflict of interest

The authors declare no conflict of interest.

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.joca.2013.01.014.

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